Transmission of tobacco rattle virus isolate PpK20 by its nematode vector requires one of the two non-structural genes in the viral RNA 2

Carmen Hernández,[1]† Peter B. Visser,[1] Derek J. F. Brown[2] and John F. Bol[1]

[1] Institute of Molecular Plant Sciences, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands
[2] Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Tobacco rattle virus isolate PpK20 is transmitted by the nematode *Paratrichodorus pachydermus*. RNA 2 of the virus determines vector transmissibility and encodes the viral coat protein and two non-structural proteins with molecular masses of 29·4 kDa and 32·8 kDa. Deletions and a frameshift in the two non-structural genes did not interfere with encapsidation or co-replication of RNA 2 with RNA 1 after mechanical inoculation of plants. Mutations that affected the 29·4K gene or both non-structural genes abolished nematode transmission, whereas a large deletion in the 32·8K gene had no effect on transmission by *P. pachydermus*. It is concluded that the 29·4K gene but not the 32·8K gene is involved in transmission of isolate PpK20 by this vector.

Tobraviruses occur naturally as isolates of tobacco rattle virus (TRV), pea early-browning virus (PEBV) and pepper ringspot virus (PRV). The genome of these viruses is bipartite and consists of single-stranded RNA of plus-strand polarity. The RNA 1 encodes functions involved in replication and cell-to-cell movement of the viral RNA (Hamilton *et al.*, 1987; MacFarlane *et al.*, 1989) whereas the RNA 2, which is variable in length and sequence, encodes the coat protein (CP) but may contain one or more additional genes for non-structural proteins, depending on the isolate (Angenent *et al.*, 1986; Goulden *et al.*, 1990; Hernández *et al.*, 1995; MacFarlane & Brown, 1995). Tobraviruses are transmitted by root-feeding *Trichodorus* and *Paratrichodorus* nematodes, and serologically distinct virus isolates have been shown to be specifically transmitted by different nematode species (Brown *et al.*, 1989). Currently, virus–vector interactions are unequivocally characterized only for the TRV isolate PpK20 transmitted by *Paratrichodorus pachydermus* and the PEBV isolate TpA56 transmitted by *Trichodorus primitivus*. In addition to the CP gene, RNA 2 of TRV-PpK20 contains reading frames for 29·4 kDa and 32·8 kDa ('29·4 K' and '32·8K') proteins (Hernández *et al.*, 1995), whereas RNA 2 of PEBV-TpA56 contains reading frames for 9K, 29·6K and 23K proteins (MacFarlane & Brown, 1995). There is no obvious sequence similarity between any of these non-structural TRV or PEBV proteins.

With pseudorecombinant viruses in which RNAs 1 and 2 of the transmissible TRV isolate PpK20 and the non-transmissible TRV isolate PLB were exchanged, it has been shown that RNA 2 determines vector transmissibility (Ploeg *et al.*, 1993). An analysis of the effects of mutations introduced into infectious

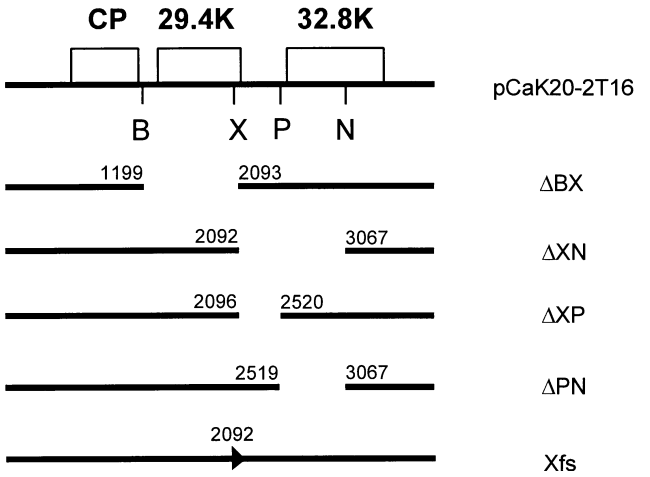

Fig. 1. Schematic representation of cDNA clones corresponding to wt RNA 2 of TRV isolate PpK20 (pCaK20-2T16) and mutants ΔBX, ΔXN, ΔXP, ΔPN and Xfs. Gaps in the bars represent deletions in the cDNAs; the numbers correspond to the nucleotides at the borders of the deletion. The frameshift in mutant Xfs is indicated by an arrowhead. The boxes represent open reading frames in the wt clone encoding the coat protein (CP), the 29·4 kDa protein ('29·4K') and the 32·8 kDa protein ('32·8K'). The positions of *Bgl*II (B), *Xba*I (X), *Pml*I (P) and *Nru*I (N) restriction sites are indicated.

Author for correspondence: John F. Bol.
Fax +31 71 5274340. e-mail j.bol@chem.leidenuniv.nl

† **Present address:** Instituto de Biología Molecular y Cellular de Plantas, Camino de Vera, 46022 Valencia, Spain

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cDNA clones of RNA 2 of PEBV isolate TpA56 and the related isolate SP5 indicated that most, if not all, of the genes in this RNA 2 play a role in vector transmission (MacFarlane *et al.*, 1995, 1996). When isolate PpK20 was serially passaged in tobacco by mechanical inoculation using leaf homogenates as inoculum, spontaneous deletion of the non-structural genes from RNA 2 was observed in some experiments and this deletion was accompanied by a loss of transmission by *P. pachydermus* (Hernández *et al.*, 1996). Here, we report that the putative RNA 2 encoded 29·4K protein but not the 32·8K protein of TRV isolate PpK20 is required for transmission of this isolate by *P. pachydermus*.

An infectious cDNA clone of RNA 2 of TRV isolate PpK20 (Hernández *et al.*, 1995) was used to engineer the mutants shown schematically in Fig. 1. In this clone (pCaK20-2T16) a full-length copy of RNA 2 (3856 nucleotides) is flanked by the CaMV 35S promoter and *nos* terminator. The positions of restriction sites in the cDNA recognized by the restriction enzymes *Bgl*II (B), *Xba*I (X), *Pml*I (P) and *Nru*I (N) are indicated in Fig. 1. Mutant ΔBX was made by digesting the plasmid at positions B and X, filling in the ends with T4 DNA polymerase and religating. Similarly, mutant ΔXN was made by digesting the plasmid at positions X and N, mutant ΔXP by digestion at positions X and P, and mutant ΔPN by digestion at positions P and N. Because *Pml*I and *Nru*I produce blunt ends, filling in with T4 DNA polymerase was omitted in the construction of mutant ΔPN. Nucleotide sequences deleted in these mutants are indicated in Fig. 1. Mutant Xfs was made by digesting pCaK20-2T16 at position X, filling in with T4 DNA polymerase and religating. This resulted in the insertion of the sequence CUAG between nucleotides 2096 and 2097 of RNA 2. The frameshift caused by this insertion results in a premature termination of the 29·4K gene reading frame in mutant Xfs. Previously, we have shown that mutants ΔBX and ΔXN were able to co-replicate at wild-type (wt) levels with RNA 1 of isolate PpK20 and are stably encapsidated after mechanical inoculation of tobacco plants (Hernández *et al.*, 1995). The same result was obtained with mutants ΔXP, ΔPN and Xfs when the corresponding cDNAs were co-inoculated with PpK20 RNA 1 on tobacco. Proper encapsidation of the mutants was confirmed by their survival in leaf homogenates incubated for 30 min at 37 °C (data not shown) and their efficient systemic spread in mechanically inoculated plants (see below).

A virus-free, mixed population of *P. pachydermus* and *T. primitivus*, in a ratio of 2 : 1, from Woodhill, Scotland, UK, was used to determine the vector transmissibility of the mutants. The virus transmission experiments were done following standard procedures (MacFarlane *et al.*, 1995) using *Nicotiana clevelandii* source and bait plants grown singly in 25 cm^3 plastic pots. Groups of 60 trichodorid nematodes were placed into each pot and an *N. clevelandii* source plant was added which, two days previously, had been mechanically inoculated with a homogenate of *N. tabacum* plants infected with RNA 1 of isolate PpK20 and a cDNA clone of RNA 2 (Hernández *et al.*, 1995, 1996). The nematodes were recovered after 4 weeks access to the roots of the virus-infected plants and added to a new pot containing a healthy *N. clevelandii* plant. After a further period of 4 weeks, the plants were removed from the pots and their roots were thoroughly washed, then triturated with a mortar and pestle. The resultant suspension was rubbed by finger onto corundum-dusted leaves of *Chenopodium quinoa* and *C. amaranticolor* virus-indicator plants. RNA was recovered from leaves of plants showing symptoms of virus infection and examined by Northern blotting to confirm the identity of the transmitted virus. The same procedure was used to confirm that the mutant viruses had invaded the root systems of the source plants.

Table 1. Effect of mutations in TRV PpK20 RNA 2 on virus transmission by *Paratrichodorus pachydermus* nematodes

Series I and II summarize two series of experiments done in different time periods. Results are shown as the number of plants with virus in root system/number of plants tested. NT, Not tested.

RNA 2 clone	Series I		Series II	
	Source plants	Bait plants	Source plants	Bait plants
pCaK20	10/10	10/10	10/10	9/10
ΔBX	10/10	0/10	NT	NT
ΔXN	10/10	0/10	NT	NT
ΔXP	3/10	NT	10/10	0/9
ΔPN	10/10	10/10	10/10	9/10
Xfs	4/10	0/4	10/15	0/10

It should be noted that each assay consisting of a group of 60 nematodes with a single source and a single bait plant represents an independent experiment. For convenience, the assays done in two different time periods are summarized as 'Series I' and 'Series II' in Table 1. Progeny of the wt clone ('pCaK20') entered the root system of all source plants inoculated, and the nematodes transmitted the virus to 10 out of 10 healthy bait plants in the first series of experiments, and to 9 out of 10 in the second. Mutants ΔBX, ΔXN and ΔPN also entered the root system of all source plants. In the first series of experiments with mutant ΔXP, and in both series of experiments with mutant Xfs, virus did not enter the roots of all source plants. Northern blot analysis of the mutant viruses in the source plants confirmed that RNA 2 of the deletion mutants had the expected length and did not hybridize to probes corresponding to the respective deletions (results not shown). Nematodes were unable to transmit mutants ΔBX, ΔXN, ΔXP and Xfs to bait plants (Table 1), but mutant ΔPN was transmitted to similar numbers of plants as the wt virus. Northern blot hybridization tests were used to confirm that the virus in these bait plants contained only RNA 2 of the size

expected for mutant ΔPN and that this RNA did not hybridize to a probe corresponding to the region deleted in this mutant.

The deletions or frameshift in the non-transmissible mutants ΔBX, ΔXN, ΔXP and Xfs affect either the 29·4K gene or both the 29·4K and 32·8K genes. In mutant ΔBX, the major part of the 29·4K gene is deleted. In mutant ΔXN, the 3′-terminal part of the 29·4K gene and the major part of the 32·8K gene are deleted. In mutant ΔXP, the 3′-terminal part of the 29·4K gene is deleted and possibly the subgenomic promoter involved in expression of the 32·8K gene is affected (Hernández *et al*., 1995). Protein expression from the wt or mutants has not been studied, but sequence analysis suggests that mutants ΔXN and ΔXP could express 29·4K proteins of which the C-terminal 5 amino acids are replaced by 3 and 15 non-viral amino acids, respectively. As a result of the frameshift in mutant Xfs, this mutant could potentially express a 29·4K protein of which the C-terminal 5 amino acids are replaced by 18 non-viral amino acids. The observation that mutant ΔPN, with a large deletion in the 32·8K gene, is fully vector transmissible demonstrates that the 32·8K gene is not required for transmission of TRV isolate PpK20 by *P. pachydermus* and implies that the non-transmissibility of the other mutants is due to their defects in the 29·4K gene. Particularly, the non-transmissibility of mutants ΔBX and Xfs shows that the 29·4K gene is essential for transmission of TRV isolate PpK20 by *P. pachydermus*. We cannot rule out the possibility that the deletions in mutants ΔBX, ΔXN and ΔXP affect RNA sequences involved in vector transmission rather than protein sequences, but the results with the frameshift mutant Xfs support the notion that vector transmission requires expression of the 29·4K gene as a functional protein.

It is possible that the role of 29·4K in nematode transmission of TRV isolate PpK20 is similar to the function of the helper component (HC) of aphid-transmissible potyviruses (Pirone, 1991) i.e. binding TRV particles to receptor sites within the vector. If so, the widely different sequences of the non-structural RNA 2 genes among tobraviruses (see Hernández *et al*., 1995; MacFarlane *et al*., 1996) could reflect specific interactions with different species of trichodorid nematodes. Although the 32·8K gene of TRV isolate PpK20 is not essential for transmission by *P. pachydermus*, it could permit transmission of this isolate by an as yet unidentified nematode vector. This hypothesis is supported by results demonstrating the involvement of CP of PEBV in transmission (MacFarlane *et al*., 1996) and by EM studies showing TRV particles present at receptor sites in *P. pachydermus*, which revealed sufficient space between the surface of the particle and the cuticle lining of the oesophageal lumen for a non-structural gene product linking the two (Brown *et al*., 1995).

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